Transcriptomic profiling of citrus fruit peel tissues reveals fundamental effects of phenylpropanoids and ethylene on induced resistance

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SUMMARY

Penicillium spp. are the major postharvest pathogens of citrus fruit in Mediterranean climate regions. Induction of natural resistance constitutes one of the most promising alternatives to avoid environmental contamination and health problems caused by chemical fungicides. To understand the bases of induction of resistance in citrus fruit against *Penicillium digitatum*, we have used a 12k citrus cDNA microarray to study transcriptional changes in the outer and inner parts of the peel (flavedo and albedo, respectively) of elicited fruits. The elicitor treatment led to an over-representation of biological processes associated with secondary metabolism, mainly phenylpropanoids and cellular amino acid biosynthesis and methionine metabolism, and down-regulation of genes related to biotic and abiotic stresses. Among phenylpropanoids, we detected over-expression of a large subset of genes important for the synthesis of flavonoids, coumarins and lignin, especially in the internal tissue. Furthermore, these genes and those of ethylene biosynthesis showed the highest inductions. The involvement of both phenylpropanoid and ethylene pathways was confirmed by examining changes in gene expression and ethylene production in elicited citrus fruit. Therefore, global results indicate that secondary metabolism, mainly phenylpropanoids, and ethylene play important roles in induction of resistance in citrus fruit.

INTRODUCTION

In nature plants are permanently in contact with a broad range of pathogens. However, disease is not widespread in plants and only a limited number of pathogens are capable of successfully invading a plant and cause disease. Plants have evolved an intricate and elaborate set of defensive barriers in order to protect themselves against pathogens (Mysore and Ryu, 2004). Preformed physical or chemical barriers constitutively present on the plant surface may initially stop the establishment of infection structures. Later on, the recognition of the pathogen may lead to activation of defence mechanisms, such as the hypersensitive response, increased expression of defence related genes like pathogenesis-related (PRs) genes, and the oxidative burst (Glazebrook, 2005, Ferreira *et al.*, 2006, Jones and Dangl, 2006, Király *et al.*, 2007). On the other hand, induced resistance activates the plant's defence mechanisms, thereby enabling the plant to better restrict the growth of a pathogen upon a subsequent attack. Although the molecular bases of induced resistance have been extensively studied in the vegetative parts of plants (Durrant and Dong, 2004, Bostock, 2005, Conrath, 2009), our knowledge of the processes underlying the establishment of induced resistance in fruits is still very poor, in most cases being limited to single metabolites, enzymes or genes. Moreover, we cannot assume that the mechanisms operating in mature fruits are equal to those found in vegetative parts of model plants. These factors strengthen the relevance of studying the mechanisms of induced resistance in crop fruits.

Penicillium digitatum (Pers.:Fr.) Sacc. is the causal agent of green mould rot, and represents the major postharvest pathogen of citrus fruit in Mediterranean regions, accounting for up to 60-80% of total losses due to fungal decay during fruit storage at ambient temperature. For many years, the control of this postharvest pathogen has mainly relied on the use of chemicals. With the current concerns about the harmful effects of synthetic fungicides on

human health and environment, there is a trend to adopt new and safer control alternatives. In citrus fruit, induction of natural resistance constitutes one of these alternatives.

Various treatments are known to trigger induced resistance in citrus fruit against fungal infections (Ben-Yehoshua *et al.*, 2000), including the application of physical (Rodov *et al.*, 1992, Droby *et al.*, 1993, Arcas *et al.*, 2000), chemical (Porat *et al.*, 2001, Porat *et al.*, 2002, Venditti *et al.*, 2005), or microbial antagonist treatments (Arras, 1996, Fajardo *et al.*, 1998, Droby *et al.*, 2002). Among these treatments, the highest induction of the antimicrobial phytoalexin scoparone was achieved in fruits subjected to pathogen inoculation followed by a heat treatment at 37ºC for 3 days (curing) (Kim *et al.*, 1991, Ben-Yehoshua *et al.*, 1992). Infected-cured citrus fruits showed a significant reduction in the incidence of green mould (Ballester *et al.*, 2010a). This treatment induced a higher level of resistance than a wounding-curing treatment, whereas curing alone increased the susceptibility to pathogen infection,

So far, the analysis of the molecular and physiological bases of induced resistance in citrus fruit has only been addressed at individual gene or enzyme activity levels. Application of elicitors, such as UV light, jasmonic acid (JA), β-amino butyric acid (BABA), wounding or brushing and hot water treatment lead to an induction of the genes coding for chitinase, β-1,3-glucanase, phenylalanine ammonia-lyase (PAL) and heat-shock proteins (Lers *et al.*, 1998, Pavoncello *et al.*, 2001, Porat *et al.*, 2001, Porat *et al.*, 2002). Induction of chitinase, β-1,3-glucanase, PAL and peroxidase activities has also been described in oranges subjected to different biotic or abiotic treatments that elicit induced resistance (Ballester *et al.*, 2010a, Fajardo *et al.*, 1998). We have recently shown that induced resistance in oranges against *P. digitatum* elicited by the combination of an inoculation with the fungus followed 1 day later by a curing treatment (37ºC for 3 days with high relative humidity) coincided with the induction of PAL, soluble peroxidase, basic β-1,3-glucanase and chitinase at both gene expression and enzyme activity levels (Ballester *et al.*, 2010a).

Ethylene may stimulate senescence, but also plays a protective role against stress conditions causing postharvest losses in citrus fruit (Lafuente and Sala, 2002, Lafuente *et al.*, 2001, Marcos *et al.*, 2005, Porat *et al.*, 1999b). Its production increases in citrus fruits infected with *P. digitatum*, being synthesized by both the fruit and the pathogen (Achilea *et al.*, 1985a, 1985b). Application of the ethylene inhibitor 1-methylcyclopropene (1-MCP) increases the susceptibility of oranges to infection by *P. digitatum* (Marcos *et al.*, 2005, Porat *et al.*, 1999b). Moreover, the fact that many genes induced in citrus fruits upon *P. digitatum* attack are also up-regulated by ethylene highlights the role of this hormone in the defence response of citrus to this pathogen (González-Candelas *et al.*, 2010). However, it is still unknown whether this hormone is also able to induce pathogen resistance in citrus fruit.

Since citrus is one of the most important and widely grown fruit crops, several genetic, genomic and proteomic tools have been quickly adopted in recent years by the citrus research community to address major challenges of this fruit crop (Talon and Gmitter Jr, 2008). The genome sequences of both *Citrus sinensis* (Sweet Orange Genome Project 2010, http:// www.phytozome.net/orange) and *Citrus clementina* (Haploid Clementine Genome, International Citrus Genome Consortium, 2011, http://int-citrusgenomics.org/, http://www.phytozome.net/clementine) have just been released. One of the strategies developed for transcriptomic analysis has been the construction of microarrays. In citrus, the first global transcriptomic study was reported by Shimada *et al*. (2005), who developed a citrus cDNA microarray containing 2,213 independent genes that was used for gene expression profiling during fruit development. The Spanish Citrus Functional Genomic Project (CFGP, http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/) has developed different generations of cDNA microarrays containing up to 20,000 probes obtained from 53 cDNA libraries covering different tissues, developmental stages and stress conditions (Forment *et al.*, 2005, Martinez-Godoy *et al.*, 2008). Two other microarrays, namely a citrus GeneChip from

Affymetrix containing 30,171 probe sets representing up to 33,879 Citrus transcripts based on Expressed Sequence Tags (ESTs) obtained from several Citrus species and Citrus hybrids, and a citrus 22k oligoarray containing 21,495 independent ESTs from Citrus species (Fujii *et al.*, 2007), have been developed recently. Currently, additional work is performed within various research groups by using large cDNA citrus microarrays or smaller custom arrays based on subtractive cDNA libraries (Bernardi *et al.*, 2010). These citrus microarrays have been used to study gene expression in relation to fruit development and quality (Bernardi *et al*., 2010, Shimada *et al*., 2005, Mayuoni *et al.*, 2011) or under various biotic (Albrecht and Bowman, 2008, Gandía *et al.*, 2007, Kim *et al.*, 2009) and abiotic stresses (Gimeno *et al.*, 2009). A first transcriptomic approach with cDNA macroarrays has recently been used to unravel the molecular processes underlying the response of citrus fruit to *P. digitatum* infection (González-Candelas *et al*., 2010). However, large scale gene expression studies addressing the process of induced resistance in citrus fruit against pathogen attack have not been conducted so far.

In this work, with the aim of better understanding the mechanisms underlying induced resistance in citrus fruit, we have carried out a large scale gene expression analysis in elicited fruits, using the 12k citrus cDNA microarray developed by the CFGP. In addition, we have examined the involvement of ethylene in the induction of resistance in citrus fruits.

RESULTS

Efficacy of elicitation of induced resistance in citrus fruits against *P. digitatum*

Combination of pathogen inoculation (denoted as I) followed by a curing treatment (3 days at 37ºC; denoted as C) reduced the incidence of a subsequent *P. digitatum* infection in oranges, with the efficacy of the treatment being dependent on the elapsed time between the curing treatment and subsequent infection (Table 1). Thus, the highest effectiveness of the infection

plus curing treatment (IC) was observed when *P. digitatum* inoculation was conducted 3 days post-treatment (dpt) (7 days after start of the experiment), although at 0 and 1 dpt (4 and 5 days after start, respectively) the incidence of the infection was also reduced as compared to non-treated fruits.

Comparative analysis of transcriptional profiles in elicited citrus fruits

A functional genomics approach employing cDNA microarrays was used to investigate the molecular responses associated with induced resistance in citrus fruits elicited by the combination of pathogen infection followed by a curing treatment. For this purpose, five different samples were analysed: non-treated 'Navelate' oranges (NT), fruits infected with *P. digitatum* during 1 day (I1), and infected-cured samples taken 4 (IC4), 5 (IC5) and 7 (IC7) days after the beginning of the experiment, thus corresponding to 0, 1 and 3 dpt, respectively. In all cases, three biological replicates of each sample, consisting each of 10 individual fruits, divided into two tissues, i.e. flavedo (F, outer-colour part of the peel) and albedo (A, inner white part of the peel), were used for transcriptomic analysis. Each sample was hybridized against a reference sample constituted by a mix of equal amounts of RNA of all the samples included in the analysis. We used the CFGP 12k microarray, which contains 24,288 clones, corresponding to 11,241 unigenes, of which 77.1% have an *A. thaliana* homolog candidate. After discarding low quality spot signals and normalization, 10,769 genes were analysed.

Fig. 1 shows a summary of the genes differentially expressed (SAM, $p<0.01$) in the flavedo and albedo of fruits subjected to the elicitor treatment compared to the non-treated fruits. In the flavedo, no single gene was differentially expressed 1 day after inoculation (FI1), whereas 299, 127 and 229 genes showed altered expression levels at 4, 5 and 7 days after the beginning of the experiment (FIC4, FIC5 and FIC7), respectively, as compared to non-treated fruits (FNT). Tables 2 and 3 show the genes with the highest induction ($\log_2 > 2$, Table 2) or

repression (log_2 < -2, Table 3) levels in the flavedo and/or albedo of elicited oranges 5 and/or 7 days after the beginning of the experiment, which are the time points with the largest reduction in disease incidence, as compared to control samples. The most highly induced genes revealed the relevance of the phenylpropanoid pathway in elicited fruits: among the 29 most up-regulated genes in the flavedo, 13 are involved in either phenylpropanoid metabolism or coumarin biosynthesis, including 7 different O-methyltransferases, an isoflavone reductase, a hydroxycinnamoyl transferase, 2 leucoanthocyanidin dioxygenases and 2 SRG1 proteins. Genes related to methionine and ethylene biosynthetic processes, such as 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), and proteins related to defence and response to stress were also induced in the flavedo (Table 2). Among the 18 most down-regulated genes in FIC5 and/or FIC7, half are involved in responses to biotic and abiotic stresses, whereas the other half have unknown functions (Table 3). In the albedo, the expression of 521, 245 and 176 genes changed significantly in AIC4, AIC5 and AIC7, respectively, as compared to ANT (Fig. 1). Similarly to the flavedo, genes related to the biosynthesis of phenylpropanoids, coumarins and methionine were highly up-regulated in AIC5 and/or AIC7 relative to ANT (Table 2). Besides these genes, the expression of different PR genes was also induced in the albedo of elicited fruits. Among the 18 genes with the highest down-regulation in the albedo of elicited fruits, there are cold-regulated genes (*COR15*) and genes with unknown function (Table 3).

Functional analysis based on the transcriptome profiles

Hierarchical cluster analysis of all differentially expressed genes (SAM, p<0.01) revealed the presence of a few major clusters containing genes that share a common expression pattern, as shown in the gene-to-gene correlation matrix (Fig. 2a). Genes within these clusters and having an *A. thaliana* homolog were subjected to a singular enrichment analysis (SEA) using

AgriGO to determine which biological processes were significantly overrepresented (Table 4). Genes included in clusters f and g, whose expression increased in response to the elicitor treatment (Fig. 2b), belonged to three major biological processes: phenylpropanoid biosynthesis, methionine metabolism and lipid biosynthesis. Most of the other pathways that appeared in these two clusters are ancestors of these three processes.

A Principal Component Analysis (PCA) was conducted in order to identify those genes that mostly accounted for the differences among tissues and treatments. The result of this analysis (Fig. 3) showed a clear difference between elicited and non-elicited tissues in the first principal component (X-axis, explaining 45.7% of the total variation). In addition, the PCA revealed a variation between the two fruit tissues, flavedo and albedo, in the second component (variation Y=19.7%) (Fig. 3a). The genes determining both tissue and treatment variations can be found by the projection of the sample differentiation vectors onto the PCA plot showing the distribution of genes (SAM analysis, $p<0.01$) (Fig. 3b).

When the two fruit tissues were subjected to PCA independently, a clear separation of samples based on treatments was observed (Figs. 3c and 3d for flavedo and albedo, respectively). Independent of treatment, 6 genes showed higher expression in flavedo than in albedo, including a putative ABC transporter, two lipoxygenases, a cinnamoyl-CoA reductase, a glucosyltransferase and a senescence-related (SRG1) protein (Table S1). Four genes were more expressed in albedo samples than in flavedo samples: two proteins without any *A. thaliana* homolog and two different Citrus Tristeza Virus (CTV) proteins (Table S2). Based on Gene Ontology (GO) terms, the biological processes over-represented in the non-treated flavedo compared to non-treated albedo are involved in biological processes associated with flavonoid and fatty acid biosynthesis (Fig. S1 and Table S3).

To elucidate key processes that were altered in elicited citrus fruits, we searched for functional enrichment categories in the set of differentially expressed genes. This analysis

showed that genes induced in the flavedo of elicited fruits (FIC5), as compared to non-treated fruits (FNT), were mainly involved in biological processes associated with secondary metabolism, in particular phenylpropanoids (Fig. 4 and Table S4). The same trend was observed in the flavedo of elicited fruits at 7 days after the beginning of the experiment (FIC7, Table S5 and Fig. S2). In the albedo, processes related to flavonoid and cellular amino acid biosynthesis and methionine metabolism were over-represented in AIC5 (Table S6 and Fig. S3), whereas two days later (AIC7) the response to cadmium ion was the most significant over-represented biological process (Table S7 and Fig. S4).

To better visualize the results of gene expression profiling experiments in a metabolic pathway context, the 'OMICS Viewer' was used. Based on the above described results two biosynthetic pathways over-represented in elicited flavedo and albedo tissues were selected for further analysis: the methionine / ethylene biosynthesis pathway (Fig. S5) and the phenylpropanoid / phenolic acid / suberin pathway (Fig. 5).

Involvement of ethylene in the induction of resistance in citrus fruits

Several genes involved in the methionine and ethylene biosynthesis pathway were up-regulated upon pathogen challenge followed by a curing treatment (Table 2 and Fig. S5). As the highest induction in the elicited flavedo (FIC5) was observed for an homolog of the *A. thaliana EFE* gene, which encodes an ACO that was 4.7-times more expressed than in FNT, we decided to study the involvement of ethylene in the fruit's response in more detail, by analysing both the expression of the *CsACO* gene and the production of ethylene in elicited fruits (Fig. 6). In non-treated fruits, *CsACO* expression was not detectable in neither flavedo nor albedo. However, in infected-cured fruits high expression was observed in both tissues, especially 5 days after the beginning of the experiment (IC5). The level of *CsACO* mRNA was 16.9- and 30.0-fold higher in the flavedo (FIC5) and albedo (AIC5) of infected-

cured fruits, respectively, compared with that of flavedo infected with *P. digitatum* during 1 day (FI1). In both tissues, the expression of *CsACO* decreased 7 days after the beginning of the experiment. This trend was also observed in the unigene aC31605B08 spotted on the 12k microarray (Table 2), which putatively also encoded another ACO. *CsACO* expression was induced in response to the wounding-curing treatment (WC7), but to a lower extent than in infected-cured fruits (IC7). On the other hand, no expression was detected in response to the curing treatment alone (C7), neither in the flavedo nor in the albedo. It is interesting to note the lack of hybridization signal with the *P. digitatum* ribosomal probe (Fig. 6a), a fact that indicates that the pathogen did not progress in the tissue that was previously inoculated with the pathogen (treatment IC).

It is known that wounding and also *P. digitatum* infection induce the expression of ethylene biosynthetic genes in the peel of *C. sinensis* fruits (Marcos *et al*., 2005). Therefore, we aimed to compare the effect of wounding and pathogen infection alone with that observed in response to the elicitor treatment, which consisted of an infection with *P. digitatum* before the curing treatment. In this experiment, 'Navelina' oranges were inoculated with 10^6 conidia mL^{-1} of *P. digitatum*, and flavedo and albedo tissues were analysed 1, 2 and 3 days after inoculation. The development of *P. digitatum* throughout both tissues was rapid, as determined by the accumulation of the *P. digitatum* 28S rRNA (Fig. 6b). Complementarily, the accumulation of the *C. sinensis* 26S rRNA was hardly detectable at 3 days post-inoculation, reflecting the degradation of fruit tissue due to fungal invasion. The pattern of *CsACO* expression was similar in flavedo and albedo. Wounded fruits showed only a small increase in *CsACO* levels 3 days after mock-inoculation in both tissues, compared to non-treated fruits. In contrast, a marked increase in *CsACO* expression was detected in both tissues during the development of infection by *P. digitatum*. The highest levels of *CsACO* mRNA

were detected at 2 days after inoculation. Nevertheless, the increase in *CsACO* expression in response to *P. digitatum* infection was lower than that observed in infected-cured fruits.

Ethylene production (Fig. 6c) was determined in discs centred on the point of inoculation. No change in ethylene production was detected in either non-treated or cured fruits, while a slight increase was detected in wounded-cured fruits 5 days after the beginning of the experiment. In contrast, high ethylene production (42 nL h^{-1} g^{-1} FW) was detected in infected-cured fruits, at day 6 of the experiment. Similar high levels were observed in infected fruits 2-3 days after inoculation with *P. digitatum*.

Involvement of the phenylpropanoid pathway in the induction of resistance in citrus fruits

It is known that genes involved in the biosynthesis of phenylpropanoids and compounds derived from this pathway are also involved in the resistance of citrus fruit against biotic and abiotic stresses (Ballester *et al.*, 2006, Kim *et al*., 1991, Sánchez-Ballesta *et al.*, 2000). Taking into account this previous result and the current transcriptomic results, we decided to conduct a deeper study on the expression of genes specifically related to the phenylpropanoid pathway. Northern blot hybridization was used both to validate the microarray results and to better define the expression patterns of selected genes putatively involved in the phenylpropanoid pathway. In total, 17 genes were analysed (Fig. 7 and Table S8) and the expression profiles for 12 of them were compared with their expression levels on the 12k microarray. Overall patterns were similar in both approaches, thus confirming our microarray results.

The expression of the 17 genes putatively involved in the phenylpropanoid pathway increased in the elicited fruits, generally reaching the highest induction levels 5 days after the beginning of the experiment. In this regard, it is noteworthy that the expression of *PAL*, the first gene in

the phenylpropanoid pathway, and also the expression of most of the O-methyltransferases analysed, increased in elicited oranges, suggesting the implication of these genes in the induction of resistance in citrus fruit. In general, the highest absolute expression levels were detected in the flavedo, while induction ratios compared to non-treated fruits were higher in the albedo.

DISCUSSION

The search for and optimization of new pathogen control systems that could help in reducing the amount of chemicals needed to control postharvest pathogens in citrus fruit will benefit from a better understanding of the biological basis of induced resistance against major pathogens. Among the different elicitor treatments to induce resistance in citrus fruits, we have chosen the combination of pathogen inoculation followed by a curing treatment as a tool to study the biological processes underlying the induction of resistance in citrus fruit in more detail. This combined treatment is highly reproducible, triggers the highest induction of the phytoalexin scoparone and leads to a higher reduction in disease incidence than wounding-curing or curing alone (Ballester *et al.,* 2010a). Building up endogenous pathogen resistance in citrus fruit by postharvest treatments is usually achieved between 1 and 3 days after elicitor treatment (Ballester *et al*., 2010a, Droby *et al*., 2002, Pavoncello *et al*., 2001). Accordingly, we have found that the combination of pathogen infection followed by a curing treatment achieves maximum efficacy at 3 days post-treatment, when a reduction of up to 70% in subsequent disease incidence was observed relative to control fruits (Table 1).

Functional genomics to elucidate induced resistance in citrus fruits

High throughput technologies have allowed a rapid progress in understanding the resistance of model plants against pathogens (De Vos *et al.*, 2005, van Loon *et al.,* 2006). Despite the

progress made in characterizing defence-related responses against *P. digitatum* infection (Angioni *et al.*, 1998, Kim *et al*., 1991, Ortuño *et al.*, 2006, Porat *et al*., 2001, Ballester *et al*., 2010a, Porat *et al.*, 1999a), remarkably little is yet known about the complex network regulating induced resistance in citrus fruit against *P. digitatum*.

P. digitatum penetrates citrus fruits through wounds that affect the albedo, the inner tissue. In more superficial wounds the chance for this pathogen to cause disease is much lower (Kavanagh and Wood, 1967). In a comparison between both tissues we found that the expression of genes involved in the phenylpropanoid pathway was higher in the flavedo (Fig. 7). However, the largest elicitation was in the albedo. Thus, the transcriptomic approach supports the idea that the flavedo is more resistant to *P. digitatum* infection than the albedo (Afek *et al.*, 1999, Ballester *et al*., 2010a, Ballester *et al*., 2006), while the internal tissue is responding more intensively to the elicitor treatment. This higher response of the albedo is also reflected in the higher number of induced/repressed genes in elicited fruits as compared to that of the flavedo (Fig. 1).

The application of different elicitors to citrus fruit has been related with the induction of genes coding for PR proteins, such as chitinases and β-1,3-glucanases (Ballester *et al*., 2010a, Porat *et al*., 2002, Porat *et al*., 2001). Indeed, a chitinase and a β-1,3-glucanase gene were induced more than 2-fold in the albedo of elicited fruits (Table 2). However, only 3 out of 9 chitinase and 3 out of 11 β-1,3-glucanase genes present on the 12k microarray were induced in the albedo, whereas the number of induced genes was even lower in the flavedo. Other PR encoding genes were also induced, such as a ribonuclease-like protein (PR-10), two hevein-like PR4 proteins and two germin-like PR16 proteins (Table 2). Notably, most of the induced PR encoding genes showed a higher induction in the albedo.

Hierarchical cluster analysis of differentially expressed genes revealed the presence of several clusters of co-expressed genes. Functional analysis of the genes included in these clusters

showed that clusters f and g, which contained up-regulated genes in both tissues at 5 days after the beginning of the experiment, were enriched in processes related to methionine metabolism and phenylpropanoid biosynthesis, and to a lesser extent to lipid biosynthesis, most specifically to isopentenyl diphosphate biosynthesis. Thus, although different factors/signals are likely involved in eliciting resistance in citrus fruit against *P. digitatum*, phenylpropanoids and ethylene seem to be the most important players in the complex network that regulates induced resistance. It is interesting to note that the metabolism of phenylpropanoids is also induced in response to *P. digitatum* infection in citrus fruits (González-Candelas *et al*., 2010).

Implication of ethylene and related hormones in induced resistance in citrus fruit

The transcriptomic analysis showed an implication of genes related to methionine and ethylene biosynthetic processes in the defence response of citrus fruit. Expression of *CsACO* was induced by the elicitor treatment (Table 2 and Fig. 6). Three additional putative *ACO* genes were present on the 12k microarray. However, only the expression of one of them (aCL3488contig1) increased in infected-cured samples. A comparative analysis of the nucleotide sequences of these four *ACO* genes showed a 29-60% identity among them (data not showed), indicating that they are different genes and not alleles of the same gene. It is well known that ethylene regulates the induction of genes related to plant defence against pathogens, including several β-1,3-glucanases and basic chitinases, PR-1 and hydroxyproline-rich proteins (Ecker, 1995). We have previously shown that the expression of genes coding for two basic β-1,3-glucanase and chitinase isoforms was induced in elicited citrus fruits (Ballester *et al*., 2010a). These previous results have been extended in the present study, where we found induction of several ethylene responsive genes, including additional β-1,3-

glucanases and chitinases, as well as other PR-encoding genes mentioned above. However many other genes encoding PR proteins were not induced by the elicitor treatment.

Infection of citrus fruit by *P. digitatum* induces ethylene production and the expression of genes involved in the synthesis of ethylene and phenylpropanoids (Marcos *et al*., 2005, Achilea *et al*., 1985a). The elicitor treatment also led to an increase in ethylene production of the same magnitude as observed in *P. digitatum*-infected fruits (Fig. 6). In infected fruits, ethylene is produced by both the fruit and the pathogen (Achilea *et al*., 1985a, Achilea *et al*., 1985b). However, in elicited fruits ethylene only originated from the fruit since the fungus did not grow within the peel, as was confirmed by the lack of hybridization with the *P. digitatum* rDNA 28S probe (Fig. 6).

Although ethylene plays a role in the resistance of citrus fruit (Marcos *et al*., 2005, Porat *et al*., 1999b), we found only a limited overlap between genes induced by the elicitor treatment and those induced by ethylene (González-Candelas *et al*., 2010). Moreover, the reduction in disease incidence achieved by ethylene treatment (Marcos *et al.*, 2005) was much lower than that observed with the infection-curing treatment, suggesting that there are other factors involved in triggering induced resistance in citrus fruits.

Ethylene and JA usually show a synergistic relationship regulating the expression of defence genes effective against necrotrophic pathogens (Glazebrook, 2005, Lorenzo *et al.*, 2003, Schenk *et al.*, 2000). The elicitor treatment altered the expression of marker genes for SA, such as PR-1, PR-2, PR5, PR-10 or class II β-1,3-glucanase, but there was not a clear trend in the observed changes, and functional analysis of differentially expressed genes did not reveal any biological process related to SA or JA in elicited citrus fruits. However, we cannot rule out the implication of these hormones in the induction of resistance of citrus.

Involvement of the phenylpropanoid pathway in the induced resistance of citrus fruits

Transcriptomic analysis revealed that the metabolism and biosynthesis of phenolic compounds are involved in the induced resistance of citrus fruits against *P. digitatum*. PAL is the first enzyme in the phenylpropanoid pathway leading to the synthesis of coumarins and flavonoids (Dixon *et al.*, 2002). Studies in citrus fruit have focused on changes in *PAL* expression in response to pathogen attack (Ballester *et al.*, 2006, McCollum, 2000) or in response to an elicitor treatment (Ballester *et al.*, 2010a, Droby *et al.*, 1993, Fajardo *et al.*, 1998). We have previously shown that *PAL* expression increased in both flavedo and albedo of infected-cured fruits. In the present work we observed that not only the expression of *PAL* but also that of a large subset of genes important for the synthesis of phenylpropanoids and flavonoids such as *C4H*, *4CL*, *COMT*, *CCoAOMT*, *CAD*, *SAD* and *POX* were increased in elicited fruits (Table 2 and Figs. 5 and 7). Transcript levels of selected phenylpropanoid genes were further investigated by Northern blot hybridization, validating the results obtained with the 12k microarray (Fig. 7). Whilst the highest expression of these genes was detected in the flavedo of the infected-cured fruits, the highest inductions were observed in the internal tissue (Fig. 7).

Not only biosynthetic genes are related to the accumulation of phenylpropanoids and flavonoids. It is well-known that R2R3-type MYB genes control many aspects of plant secondary metabolism (Stracke *et al.*, 2001). It has been recently described that the lack of expression of the *MYB12* transcription factor, which controls the expression of the biosynthetic flavonoid genes, is related with the lack of accumulation of naringenin chalcone in tomato (Adato *et al.*, 2009, Ballester *et al.*, 2010b). The *A. thaliana MYB12* homologue was included in the 12k microarray, but it was not affected by the elicitor treatment. However, two MYB, two WRKY and one bHLH transcription factors clustered within the e, f and g groups

in the gene-to-gene correlation analysis. Additional analysis will be addressed in order to clarify the possible role of these transcription factors in the induced response.

In conclusion, the transcriptomic analysis of elicited oranges showed important up-regulation in the expression of genes involved in the metabolism of phenylpropanoids and synthesis of ethylene and down-regulation of genes related to diverse biotic and abiotic stresses. Our results indicate that the highest inductions were found in the albedo, whereas the highest expression values were detected in the external tissue. These results reinforce the idea that the internal tissue is more susceptible to *P. digitatum* infection and it is the one that should increase to a greater extent the defensive barriers in order to avoid the progression of the fungus. To the best of our knowledge this is the first study in any harvested fruit that has addressed the analysis of global changes in gene expression in the process of induced resistance.

EXPERIMENTAL PROCEDURES

Fruit and fungal material

Oranges (*Citrus sinensis* L. Osbeck) from a commercial orchard in Lliria (Valencia, Spain) were selected and used in the experiments before any commercial postharvest treatment was applied. 'Navelate' fruits were taken in three independent samplings and used for the induction of resistance treatment, and 'Navelina' oranges were employed to study the expression of *CsACO* during the infection of *P. digitatum*. Fruits were immediately surface-sterilized with 5% commercial bleach solution for 5 min, extensively washed with tap water and allowed to dry at room temperature until next day.

Petri dishes containing potato dextrose agar were inoculated with *Penicillium digitatum* (Pers.:Fr.) Sacc. isolate PHI-26 and incubated at 24ºC for 7 days (López-García *et al.*, 2000). Conidia were rubbed from the agar surface by scraping them with a sterile spatula and

transferred to sterile water. The conidial suspension was then filtered and the concentration determined with a haemocytometer and adjusted to the desired concentration.

Induction of resistance treatment and *P. digitatum* infection

The treatment for eliciting resistance was described previously by Ballester *et al*. (2010a). Briefly, the following treatments were applied on the three biological replicates of 'Navelate' oranges: (i) fruits were wounded by making punctures (3 mm in depth) with a sterilized nail and inoculated with 10 µL of a *P. digitatum* conidial suspension adjusted to 10^5 conidia mL^{-1}. Treated fruits were placed into plastic boxes and maintained at 90-95% relative humidity (RH) and 20ºC for 1 day to allow pathogen development. Then, fruits were heat-treated at 37ºC for 3 days under water-saturated conditions (curing) in order to stop the progress of the pathogen (Sample IC); (ii) control inoculations were carried out by injecting 10 µl of sterile water and holding the fruits under the same conditions (Sample WC); (iii) additional controls consisted of intact non-wounded fruits held at 20ºC for 1 day and then at 37ºC for 3 days (Sample C) and intact non-wounded fruits held at 20ºC for 1 day and then at 4ºC for 3 days (Sample X). A sample from intact non-wounded fruits was obtained the first day of the experiment (Sample NT). Peel tissue discs of 13 mm around the inoculation point were sampled using a cork borer. Flavedo and albedo tissues were separated with a scalpel. Tissue discs obtained from 15 oranges with 8 discs per fruit were immediately frozen in liquid nitrogen, mixed and grounded to a fine powder with a coffee mill and stored at -80ºC until further analysis.

To check the influence of *P. digitatum* infection in the expression of *CsACO*, 'Navelina' oranges were wounded by making punctures (5 mm in depth) with a sterilized nail and inoculated with 10 µL of a suspension of *P. digitatum* conidia adjusted to 10^6 conidia mL^{-1}. This high inoculum level was used in order to synchronize fungal development in all wounds.

Oranges were kept at 20ºC up to 3 days (Samples I). As controls, wounded fruits inoculated with sterile water (Samples W) or non-wounded fruits (Samples NT) were also taken. At 1, 2 and 3 days post-inoculation, flavedo and albedo discs of 7 mm around the point of inoculation were sampled by using a cork borer. Flavedo and albedo tissues were processed as described above and stored at -80ºC until RNA isolation.

Infections

To determine the effectiveness of the elicitor treatment reducing pathogen infection and the importance of the elapsed time between the treatment and the ulterior infection, disease susceptibility was analysed at the beginning of the experiment in non-treated 'Navelate' fruits and at 4, 5 and 7 days (0, 1 and 3 dpt) for the infected-cured fruits. Each infected-cured fruit was punched at a distance of 0.5 cm from the previous wound or in the equatorial axis in fruits that had not been previously inoculated. Then, 10 µL of a 10^4 conidia mL^{-1} suspension of *P. digitatum* spores were applied to each wound. After inoculation, fruits were kept at 20ºC and 90-95% RH. The incidence of infection, as a percentage, was determined for up to 6 days of incubation at 20ºC. The experimental design consisted of 3 replicates of 5 fruits, with 4 wounds per fruit, for each treatment. To test the effect of the treatments, a one-way analysis of variance (ANOVA) was performed. Means were separated using the Tukey's Honestly Significant Difference test at $p<0.05$. The analysis was performed with Statgraphics Plus 5.1 Software (Manugistics, Inc.).

RNA isolation and preparation of labelled cDNA probes

Total RNA was isolated from frozen tissue as described by Ballester *et al*. (2006). RNA concentration was measured spectrophotometrically and the integrity was verified by agarose gel electrophoresis and ethidium-bromide staining.

RNA samples for microarray hybridizations were labelled with the indirect method, by incorporation of 5-(3-aminoallyl)-2-deoxy-UTP (aa-dUTP) into single-stranded cDNA during reverse transcription, followed by conjugation of fluorescent Cy3 and Cy5 as reactive N-hydroxyl succinimidal dyes (NHS-dyes). Reverse transcription, cDNA purification, dye coupling, and fluorescent cDNA purification were accomplished as described by Forment *et al*. (2005), except that total RNA (30 µg) was used instead of poly(A)+ RNA. Sample RNA was labelled with Cy5, and reference RNA (pooled RNA consisting of an equal amount of RNA from each sample) was labelled with Cy3.

Microarray hybridization, data acquisition and data analysis

The 12k cDNA microarray developed by the Citrus Functional Genomic Project (CFGP; http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/) was used. This microarray contains probes corresponding to 11,241 putative unigenes from citrus. Microarray hybridization, washing and scanning was performed as described by Forment *et al*. (2005), with some modifications. Labelled cDNA from experimental and control samples were dried separately and resuspended in fresh hybridization solution [containing 50% (v/v) formamide, 5x SSC, 0.1% (w/v) SDS and 0.1 mg mL^{-1} salmon sperm DNA]. Samples were heated for 1 min at 95ºC before hybridization, which was conducted at 42°C. Microarray slides were scanned with a GenePix 4000B (Axon Instruments, Sunnyvale, CA, USA) using 'GenePix Pro 6.0' image acquisition software (Axon Instruments, Sunnyvale, CA, USA) at 10 µm resolution, 100% laser power, and different photomultiplier tube (PMT) values to adjust the channels intensity ratio to 1.0. Non-homogeneous and aberrant spots were discarded. Only spots with background-subtracted intensity greater than 2-fold the mean background intensity in at least one channel were selected and used for normalization and further analysis. Data were log transformed and analysed using GEPAS (Gene Expression Pattern Analysis Suite) software

v3.1 (Montaner *et al.*, 2006). Firstly, the DNMAD module (Diagnosis and Normalization for MicroArray Data) (Vaquerizas *et al.*, 2004) was used to normalize the data using background subtracted median values and an intensity-based Lowess function within and among microarrays. The Preprocessing application included in GEPAS (Herrero *et al.*, 2003), was used to merge gene replicates values. Finally, genes detected in only one of the three biological replicates were discarded.

Identification of differentially expressed genes was done using SAM (Significant Analysis of Microarrays) (Tusher *et al.*, 2001) included in the TM4 Microarray Software Suite (Saeed *et al*., 2003). Genes that satisfied the statistical threshold (False Discovery Rate, adjusted *p*-values<0.01) were considered as differentially expressed. Multivariate analysis including hierarchical cluster analysis and Principal Component Analysis (PCA) were performed using the GeneMaths XT software package (http://www.applied-maths.com/). Pearson's product-moment correlation coefficient was used as a measure for gene-to-gene correlation.

AgriGO (Zhou and Su, 2007) was used to extract Gene Ontology (GO) terms that were significantly over- or under-represented in a particular set of genes relative to a reference group composed of all genes present in the microarray which have an *Arabidopsis thaliana* homologue. To overlay the gene expression data derived from the microarray hybridizations on a metabolic map, the OMICS Viewer tool from AraCyc 3.5 was used (Mueller *et al.*, 2003).

Northern blot analysis

Northern blot analysis was carried out by electrophoresis of denatured total RNA (10 µg) in 1.2% (w/v) agarose-formaldehyde gel and blotted onto nylon Hybond-N^+ membrane (Amersham-Bioscience). cDNA labelling, hybridization and quantification were carried out as described previously by Ballester *et al*. (2006). Probes used for Northern hybridization were

obtained from different cDNA libraries previously generated in our group (Table S8): (i) RindPdig24 cDNA library, which is derived from 'Clemenules' mandarins infected with *P. digitatum* (Forment *et al*., 2005, González-Candelas *et al*., 2010), (ii) FlavCurFr1 derived from heat-treated 'Fortune' mandarins (Forment *et al*., 2005) and (iii) RindPdigS, a subtractive cDNA library constructed from the peel of 'Navelina' oranges infected with *P. digitatum* (González-Candelas *et al*., 2010).

For normalization, filters were hybridized to the 26S rDNA *C. sinensis* probe (Ballester *et al*., 2006). With few exceptions, for each gene a value of 1.0 was assigned to the normalized signal of non-treated flavedo and the expression level of the rest of the samples referred to it. After stripping the blots they were hybridized using 28S rDNA *P. digitatum* probe (Ballester *et al*., 2006).

Ethylene production

Ethylene production from 10 mm discs obtained with a cork bored around the point of inoculation was determined by incubating the discs in sealed glass tubes at 20ºC. After 15 min of incubation at this temperature, a 1 mL of headspace gas sample was withdrawn from each tube and analysed as described by Lafuente *et al*. (2001) with a Perkin Elmer gas chromatograph (GC) (Norwalk, CT) equipped with a flame ionization detector and an alumina column (1 m x 2 mm diameter, 80/100 mesh) from Supelco (Barcelona, Spain). Nitrogen was used as carrier gas and the temperature of the column was maintained at 140ºC. Ethylene standard was obtained from Abello-Oxígeno-Linde S. A. (Valencia, Spain). The results are the mean of three replicate samples of 9 discs from 3 different oranges.

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SUPPORTING INFORMATION LEGENDS

Table S1. List of genes showing higher expression in flavedo than in albedo in all five situations analysed according to SAM (p<0.01).

Table S2. List of genes showing higher expression in albedo than in flavedo in all five situations analysed according to SAM (p<0.01).

Table S3. List of genes showing higher expression in non-treated flavedo (FNT) than in non-treated albedo (ANT). Those genes with an *A. thaliana* homolog were used to generate the hierarchical view of the GO (Fig. S1).

Table S4. List of genes showing higher expression in elicited flavedo 5 days after the beginning of the experiment (FIC5) than in non-treated flavedo (FNT). Those genes with an *A. thaliana* homolog were used to generate the hierarchical view of GO (Fig. 4).

Table S5. List of genes showing higher expression in the elicited flavedo 7 days after the beginning of the experiment (FIC7) than in non-treated flavedo (FNT). Those genes with an *A. thaliana* homolog were used to generate the hierarchical view of GO (Fig. S2).

Table S6. List of genes showing higher expression in elicited albedo 5 days after the beginning of the experiment (AIC5) than in non-treated albedo (ANT). Those genes with *A. thaliana* homolog were used to generate the hierarchical view of GO (Fig. S3).

Table S7. List of genes showing higher expression in elicited albedo 7 days after the beginning of the experiment (AIC7) than in non-treated albedo (ANT). Those genes with an *A. thaliana* homolog were used to generate the hierarchical view of GO (Fig. S4).

Table S8. Genes analysed by Northern blot hybridization.

Fig. S1. Hierarchical view of Gene Ontology (GO) biological process categories significantly over-represented in the non-treated flavedo (FNT) compared to non-treated albedo (ANT) using AgriGO.

Fig. S2. Hierarchical view of Gene Ontology (GO) biological process categories significantly over-represented in elicited flavedo 7 days after the beginning of the experiment (FIC7) compared to non-treated flavedo (FNT) using AgriGO.

Fig. S3. Hierarchical view of Gene Ontology (GO) biological process categories significantly over-represented in elicited albedo 5 days after the beginning of the experiment (AIC5) compared with non-treated albedo (ANT) using AgriGO.

Fig. S4. Hierarchical view of Gene Ontology (GO) biological process categories significantly over-represented in elicited albedo 7 days after the beginning of the experiment (AIC7) compared with non-treated albedo (ANT) using AgriGO.

Fig. S5. Graphic representation of the methionine and ethylene biosynthesis pathways showing gene expression values in the flavedo (F) and albedo (A) of elicited oranges. In light blue colour are indicated genes with differential expression according to SAM analysis. The first number in brackets indicates the number of genes spotted into the microarray that have an *A. thaliana* homolog, whereas the second number indicates the total number of *A. thaliana* genes for each step in the pathway. Numbers in squares indicate the log2 ratio of: A, I1/NT; B, IC4/NT; C, IC5/NT and D, IC7/NT. nd: non-detected. The symbol + indicates no expression in NT, and the symbol -, expression in NT fruits but not in the compared treatment. Abbreviations: NT: Non-treated fruits. I1: infected fruits 1 day after pathogen inoculation. IC: infected and cured fruits at 4, 5 or 7 days after the beginning of the experiment.

FIGURE LEGENDS

Fig. 1. Summary of differentially expressed genes (SAM, $p<0.01$) in the flavedo (grey bars) and albedo (white bars) of fruits infected with *P. digitatum* during 1 day (I1) and infected and cured fruits at 4 (IC4), 5 (IC5) and 7 (IC7) days after the beginning of the experiment

compared to non-treated (NT) fruits. Genes differentially expressed in both tissues are represented as stripped bar.

Fig. 2. Gene-to-gene correlation matrix of differentially expressed genes. (a) Main gene clusters are situated along the diagonal line (groups a–g). Correlations between genes are shown in blue scale: the darker the blue colour, the higher the percentage of similarity between gene expression patterns. (b) Patterns of expression of flavedo (F) and albedo (A) genes included in each cluster. Abbreviations: NT non-treated fruits; I1 fruits infected with *P. digitatum* during 1 day; IC infected and cured fruits at 4, 5 and 7 days after the beginning of the experiment.

Fig. 3. Multivariate analysis of differentially expressed genes in elicited citrus fruits. (a) PCA showing the variation between fruits subjected or not to the elicitor treatment in the first, and the variation between tissues, flavedo (F) and albedo (A), in the second component. (b) PCA showing the distribution of genes. Genes with a log2 ratio expression >2 for induced and <-2 for repressed are indicated in different colours. Independent PCAs for the flavedo (c) and albedo (d) samples. See nomenclature in Figure 1.

Fig. 4. Hierarchical view of Gene Ontology (GO) biological categories significantly over-represented in the elicited flavedo 5 days after the beginning of the experiment (FIC5) compared to non-treated flavedo (FNT) obtained with AgriGO. Significant categories (adjusted *p*-value <0.05) are shown using a colour scaling according to their significance level. Other categories required to complete the hierarchy are shown in grey.

Fig. 5. Graphical representation of the phenylpropanoid, free phenolic acids and suberin pathways showing gene expression values in the flavedo (F) and albedo (A) of elicited oranges. In light blue colour are indicated genes with differential expression according to SAM analysis. The first number in brackets indicates the number of genes spotted into the microarray that have an *A. thaliana* homolog, whereas the second number indicates the total number of *A. thaliana* genes for each step in the pathway. Numbers in squares indicate the log2 ratio of: a, I1/NT; b, IC4/NT; c, IC5/NT and d, IC7/NT. nd: non-detected. The symbol + indicates no expression in NT, and the symbol -, expression in NT fruits but not in the compared treatment. Abbreviations: NT, non-treated fruits. I1, fruits infected with *P. digitatum* during 1 day; IC, Infected and cured fruits at 4, 5 or 7 days after the beginning of the experiment

Fig. 6. Involvement of ethylene in the response of citrus fruits to the elicitor treatment and in response to *P. digitatum* infection. (a) Northern blot analysis of *CsACO* in the flavedo and albedo of oranges: NT, non-treated; I, infected; IC, infected and cured; X, non-wounded; C, cured; WC, wounded and cured fruits. (b) *CsACO* mRNA accumulation in oranges infected with *P. digitatum* during 1, 2 and 3 days. Fruits were either non-treated (NT), wounded and water inoculated (W), or wounded and inoculated with a suspension of 106 conidia mL-1 of *P. digitatum* (I). In panels (a) and (b), relative accumulation (R.A.) values of *CsACO* mRNA in arbitrary units are shown at the bottom. Normalization was carried out with respect to the hybridization signal of the *C. sinensis* 26S rRNA using the non-treated flavedo as a reference. Hybridization with the *P. digitatum* 28S rDNA probe is shown at the bottom. (c) Ethylene production in discs of non-treated oranges (●), infected fruits (⬡), cured fruits (△), wounded and cured fruits (□), infected and cured fruits (◇).Values represent the average of three replicates, including 10 discs in each replicate, ± SD.

Fig. 7. mRNA relative accumulation of genes putatively involved in the phenylpropanoid metabolism in elicited citrus fruits. The expression level was determined by Northern blot (●) or microarray (□) hybridizations. In general, a reference value of 1.0 was assigned to the non-treated flavedo, except for *COMT1* and *SAD* genes due to lack of expression in non-treated flavedo. Values from microarray hybridizations represent the average of three biological replicates ± SD. Abbreviations: NT non-treated fruits; I1 fruits infected with *P. digitatum* during 1 day; IC infected and cured fruits at 4, 5 and 7 days after the beginning of the experiment.

Table 1. Incidence (percentage of infection) of green mould disease caused by *P. digitatum* in elicited 'Navelate' oranges. Wounded fruits were inoculated with 10 µL of a *P. digitatum* spore suspension containing 10^5 conidia mL^{-1} and incubated for 24 h at 20ºC and then for 3 d at 37ºC and 90-95% RH. Non-treated oranges were included as a control. At 0, 1 or 3 days post-treatment (dpt) (4, 5 or 7 days after the beginning of the experiment, respectively) fruits were inoculated with 10 µL of a *P. digitatum* spore suspension containing 10^4 conidia mL^{-1}. Inoculation sites were 0.5 cm apart from the previous wound in the infected-cured fruits. Incidence was determined for up to 6 days of incubation at 20ºC following inoculation. Data shown correspond to 6 days post-inoculation. Different letters indicate significant differences in the treatments according to Tukey test with a *p*-value of 0.05.

	days	dpt	Incidence (%)
Non-treated fruits	0		86.7 a
Infection + Curing	4	0	61.9 b
	5	1	50.0 c
	7	3	25.7 d

Table 2. Citrus genes with the highest induction level (log_2> 2) in the flavedo and/or albedo of elicited oranges 5 and/or 7 after the beginning of the experiment. Values represent the log_2 ratio of: a, I1/NT; b, IC4/NT; c, IC5/NT and d, IC7/NT. Numbers in bold indicate differential expression in the compared conditions according to SAM (*p*-value<0.01) and log_2> 2. The symbol + indicates no expression in NT, and the symbol -, expression in NT fruits but not in the compared treatment. NT: Non-treated fruits. I1: infected fruits 1 day after pathogen inoculation. IC4: infected and cured fruits 4 days after the beginning of the experiment. IC5: infected and cured fruits 5 days after the beginning of the experiment. IC7: infected and cured fruits 7 days after the beginning of the experiment.

Citrus unigene	Description	Arabidopsis homolog	Flavedo				Albedo			
			a	b	c	d	a	b	c	d
Phenylpropanoid and flavonoid biosynthetic process										
aCL3343Contig1	Caffeic acid 3-O-methyltransferase	AT5G54160	0.23	**2.38**	**4.22**	**3.18**	0.28	**2.47**	**4.18**	**3.41**
aCL38Contig7	Catechol O-methyltransferase	AT5G54160	0.37	1.17	3.56	1.78	0.09	1.66	**3.86**	**2.49**
aC06052D07T7	Caffeic acid 3-O-methyltransferase 1	AT5G54160	-0.82	1.45	**3.58**	1.92	0.56	**2.14**	**3.83**	**3.08**
aCL38Contig8	Eugenol O-methyltransferase	AT5G54160	-0.41	**2.29**	**3.43**	**3.28**	0.74	**2.39**	**3.79**	**4.04**
aC08010D03SK	Caffeic acid O-methyltransferase	AT5G54160	-0.53	1.53	**3.41**	**2.08**	0.03	1.36	**3.36**	2.10
aC31502H09EF	SRG1 protein	AT1G17020	0.37	0.49	2.36	**2.85**	0.55	1.33	**2.84**	**3.40**
aCL38Contig2	Phloroglucinol O-methyltransferase	AT5G54160	-0.18	1.12	3.81	-	-	0.37	**2.85**	-
aCL3152Contig1	Hydroxycinnamoyl transferase	AT5G48930	-0.03	1.14	**2.23**	1.51	0.79	1.67	**2.60**	2.27
aC08017C07SK	Isoflavone reductase-like protein	AT1G19540	-0.79	1.20	**2.37**	0.94	0.36	1.17	**2.34**	1.26
aC31207A03EF	Eugenol O-methyltransferase	AT5G54160	0.20	1.11	**2.26**	**2.02**	0.43	0.95	1.79	0.85
aCL5465Contig1	SRG1 protein	AT1G17020	0.45	0.51	1.26	**2.21**	0.63	1.56	1.63	2.24
aCL1474Contig1	Cinnamyl alcohol dehydrogenase	AT5G19440	0.66	1.01	1.35	1.60	0.64	1.80	1.82	**2.20**
aC31705B10EF	Eugenol O-methyltransferase	AT5G54160	-0.13	1.03	**2.14**	1.55	0.43	0.24	1.18	0.98
aC31807C06EF	Acridone synthase II	AT5G13930	0.51	0.81	1.63	2.22	0.72	1.32	0.85	1.63
Coumarin biosynthetic process										
aC31108D04EF	Leucoanthocyanidin dioxygenase-like protein	AT3G13610	-1.21	**3.08**	**3.77**	**3.96**	0.49	**3.46**	**4.36**	**4.26**
aCL18Contig10	Caffeoyl-CoA O-methyltransferase 2	AT4G34050	0.13	1.61	2.24	1.79	0.69	**2.99**	**4.16**	**3.33**
acl7037Contig1	Caffeoyl-CoA O-methyltransferase	AT4G34050	0.22	0.48	1.30	0.59	0.02	1.33	**2.32**	1.68
aCL139Contig2	Caffeoyl-CoA O-methyltransferase 2	AT4G34050	0.14	-0.26	1.03	-0.36	0.56	0.93	**2.24**	0.94
aCL8378Contig1	Leucoanthocyanidin dioxygenase-like protein	AT3G13610	-	1.53	**2.22**	-		+	+	+
Methionine and ethylene biosynthetic processes										
aC31605B08EF	ACC oxidase	AT1G05010	0.24	2.24	4.22	1.47	1.61	**2.76**	**4.70**	2.68
aCL3488Contig1	ACCO2	AT2G19590	-	0.85	**2.48**	-	0.03	0.66	1.03	0.35
aCL90Contig4	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	AT5G17920	-0.07	0.41	**2.13**	0.16	-0.22	0.73	**2.09**	0.36

Cont. Table 2

Citrus unigene	Description	Arabidopsis homolog	Flavedo a	Flavedo b	Flavedo c	Flavedo d	Albedo a	Albedo b	Albedo c	Albedo d
Defence related proteins and Response to stress										
aCL46Contig3	PR10A	AT1G24020	0.87	1.56	1.09	1.31	1.27	**3.61**	**3.45**	**3.03**
aCL1Contig14	PR-4A precursor	AT3G04720	-0.50	1.47	1.34	**2.04**	-0.21	**2.06**	**2.42**	**3.09**
aCL7008Contig1	Germin-like protein subfamily 1 member 13 precursor	AT5G39120	1.51	3.26	2.39	2.69	1.31	**3.72**	**3.06**	**3.05**
aCL311Contig2	Germin-like protein subfamily 1 member 13 precursor	AT5G39150	1.70	2.24	2.31	2.68	1.03	**3.67**	**3.02**	**2.96**
aCL3449Contig1	Dicyanin	AT5G20230	0.51	1.22	2.12	1.51	1.27	1.89	**3.01**	**2.47**
aCL2358Contig2	Putative embryo-abundant protein	AT2G41380	-0.98	0.45	1.69	0.23	1.06	1.66	**2.87**	**2.04**
aCL3319Contig1	Chitinase CHI1	AT3G54420	-1.03	1.86	1.61	1.40	0.68	**2.38**	**2.06**	**2.82**
aCL20Contig7	β-1,3-glucanase precursor	AT3G57270	-0.33	-0.40	1.61	0.35	-0.61	0.68	**2.81**	1.18
aCL9Contig16	Lea5	AT4G02380	0.07	1.99	1.25	**2.31**	-0.41	0.95	0.50	0.90
aCL9353Contig1	Homogentisate phytylprenyltransferase (HPT1)	AT2G18950	1.03	1.59	1.95	1.63	1.20	**2.13**	**2.28**	**2.05**
aC34104H09EF	Receptor kinase Lecrk	AT2G37710	0.10	1.10	1.69	1.01	0.17	1.25	**2.13**	0.98
aC18021E12Rv	Isoleucine-tRNA ligase-like protein	AT4G10320	0.51	1.20	0.86	1.05	1.03	**2.07**	**2.10**	1.89
aC31304H09EF	Putative glutathione S-transferase T3	AT3G09270	-0.15	0.95	2.07	1.55	0.36	1.15	1.99	**2.13**
aCL7071Contig1	PR4b	AT3G04720	-0.56	1.24	1.25	1.57	0.41	1.54	1.62	**2.24**
aCL5030Contig1	PR5-1	AT4G11650	-0.20	1.58	0.96	1.49	-0.27	1.39	1.53	**2.04**
aCL9Contig11	Lea5		-0.02	1.84	1.19	**2.02**	-0.31	0.95	0.55	0.93
Others										
aC34005H07EF	Expressed protein	AT3G48770	-0.09	1.58	**2.28**	1.39	0.66	**3.02**	**4.06**	**3.32**
aCL2Contig10	No annotation available		0.05	1.65	1.97	**3.69**	0.16	0.85	1.49	**2.75**
aC05803G03SK	No annotation available		0.20	0.67	2.00	-0.40	1.83	1.81	**3.69**	1.72
aC31305B02EF	No annotation available		0.11	1.40	1.54	**3.34**	0.68	1.51	**2.07**	**3.50**
aC08012E03SK	No annotation available		0.06	1.45	1.29	**3.04**	-0.05	1.03	1.94	**3.48**
aCL257Contig1	Putative DNA binding protein	AT4G27000	0.06	2.16	2.28	1.68	0.53	**2.59**	**3.29**	**2.48**
aCL8302Contig1	No annotation available		-1.00	1.07	1.03	1.52	0.62	**2.17**	**2.37**	**3.20**
aCL5505Contig1	Beta-cyanoalanine synthase	AT3G61440	-0.51	-0.30	2.72	1.70	0.14	0.74	**3.16**	2.23
aCL363Contig1	Expressed protein	AT4G32480	-0.26	0.90	2.49	0.47	0.98	1.63	**3.08**	1.76
aCL5472Contig1	No annotation available		0.06	0.13	1.51	-0.30	1.39	1.23	**2.93**	1.24
aCL7118Contig1	FAD-binding domain-containing protein	AT4G20820	0.29	1.12	1.99	1.06	1.25	**2.18**	**2.77**	2.06
aC08027C08SK	Agglutinin-2 precursor	AT5G65600	-0.62	1.53	2.42	0.93	-0.07	1.88	**2.74**	1.74
aCL8719Contig1	Globulin-like protein	AT1G07750	-0.27	-	-0.20	0.64	0.92	-	0.79	**2.73**
aC05811H08SK	Transferase family protein	AT1G28680	0.03	0.71	1.82	0.59	0.62	1.46	**2.45**	1.16
aCL3612Contig1	Cytochrome P450 79A2	AT5G05260	-0.53	0.41	1.31	0.73	0.59	1.49	**2.44**	1.73
aC08016G11SK	No annotation available		0.27	1.00	0.97	**2.37**	0.13	0.24	0.66	1.92
aCL214Contig1	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 3	AT1G22410	-0.09	0.27	1.25	-0.10	0.86	1.53	**2.36**	0.94
aC01008H04SK	Phosphatidate cytidylyltransferase family protein	AT3G45040	0.37	1.23	1.94	0.53	0.19	1.45	**2.30**	0.79
aCL1084Contig1	FAD-binding domain-containing protein	AT4G20820	0.62	1.39	2.26	1.48	0.56	1.65	**2.17**	1.51
aCL944Contig2	EIG-I24 protein	AT1G28680	0.80	1.07	**2.16**	0.85		+	+	
aC08019H09SK	No annotation available		0.30	1.39	0.93	**2.14**	0.29	1.02	0.90	**2.05**
aCL866Contig1	CYP81E8	AT4G37370	1.09	1.46	1.59	**2.05**	0.22	0.69	1.01	1.08
aC18010B08Rv	No annotation available		0.11	0.72	2.30	0.40	0.42	0.74	**2.11**	0.78
aCL632Contig3	Proline-rich protein	AT4G38770	0.12	1.14	1.48	0.20	0.44	1.41	**2.08**	1.00

Cont. Table 2

Citrus unigene	Description	Arabidopsis homolog	Flavedo				Albedo			
			a	b	c	d	a	b	c	d
aCL1591Contig2	Expressed protein	AT5G65520	-	-	1.90	1.37	0.30	0.67	**2.07**	1.49
aCL3641Contig1	Ripening regulated protein DDTFR18	AT5G17700	-0.11	1.98	1.14	**2.03**	-0.43	1.28	0.73	0.92
aCL8293Contig1	CYP82C1p	AT4G31940	0.75	1.69	1.45	**2.03**	0.34	1.16	0.81	1.16
aC19007C04T7	No annotation available		-0.11	1.84	1.12	**2.03**	-0.49	0.94	0.46	0.84

Table 3. Citrus genes with the highest repression level (log_2 < -2) in the flavedo and/or albedo of elicited oranges 5 and/or 7 after the beginning of the experiment. Values represent the log_2 ratio of: a, I1/NT; b, IC4/NT; c, IC5/NT and d, IC7/NT. Numbers in bold indicate differential expression in the compared conditions according to SAM (*p*-value<0.01) and log_2 < -2. The symbol – indicates expression in NT fruits but not in the compared treatment. See nomenclature in Table 2.

Citrus unigene	Description	Arabidopsis homolog	Flavedo				Albedo			
			a	b	c	d	a	b	c	d
Defence related proteins and Response to stress										
aCL2916Contig1	Probable polygalacturonase non-catalytic subunit JP650 precursor	AT1G70370	0.55	-2.01	**-2.72**	**-3.38**	0.92	-1.77	-	-2.91
aCL6368Contig1	Probable polygalacturonase non-catalytic subunit JP650 precursor	AT1G70370	0.84	-1.65	**-2.64**	**-3.14**	0.98	-1.54	-	-
aCL5Contig15	Putative early light induced protein	AT3G22840	-0.85	**-2.77**	**-2.36**	**-3.10**	-0.43	-0.70	-0.84	-1.53
aCL80Contig2	Chloroplast small heat shock protein class I	AT3G46230	-0.07	0.97	-0.65	-1.87	-0.38	0.01	-1.60	**-3.08**
aCL2349Contig1	Putative beta-1,3-glucanase	AT2G16230	0.23	-1.46	-1.87	**-2.83**	0.30	0.21	-0.54	-0.98
aC31403B04EF	Dehydrin family protein	AT1G54410	-0.18	**-2.88**	**-2.07**	-1.27	-0.43	**-3.13**	**-2.80**	-1.60
aCL6Contig15	Dehydrin family protein	AT1G54410	-0.97	**-3.60**	**-2.71**	-1.98	-0.44	**-3.04**	**-2.57**	-1.56
aC31502B11EF	Plasma membrane intrinsic protein	AT4G00430	0.35	-1.36	**-2.33**	-1.96	1.20	0.41	-1.25	-1.03
aC20006B02SK	MYB91	AT2G37630	-0.28	**-2.29**	**-2.07**	-1.32	-0.44	**-2.29**	-	-1.27
Others										
aCL2649Contig1	No annotation available		-0.77	-	-	-	-0.64	-1.26	-2.42	**-3.36**
aCL1642Contig3	No annotation available		-0.51	**-2.14**	-1.82	**-3.23**	-0.76	-1.29	-2.06	-2.66
aCL3246Contig1	Steroid sulfotransferase-like protein	AT5G07010	0.37	-1.73	-	**-3.26**	0.92	0.32	-0.44	-1.48
aC20005G09SK	Importin beta-2	AT5G53480	0.32	-1.52	-1.84	**-3.17**	0.11	0.35	0.03	-0.83
aCL4849Contig1	Expressed protein	AT5G01750	-0.20	0.92	-0.76	-1.84	-0.42	-0.44	-1.46	**-3.10**
aCL6348Contig1	T2J13.20 protein	AT3G59300	-0.36	**-2.71**	**-2.01**	-1.21	-0.32	**-2.97**	**-2.70**	-1.75
aC06003B11SK	Expressed protein	AT5G14790	-0.26	**-2.69**	**-2.01**	-1.24	-0.23	**-2.75**	**-2.54**	-1.53
aCL6Contig6	No annotation available	AT1G54410	-0.33	**-2.56**	**-2.04**	-1.30	-0.34	**-2.56**	**-2.49**	-1.63
aC08006G03SK	Proline-rich extension-like family protein	AT1G21310	0.05	-0.88	-1.83	**-2.44**	0.18	-0.61	-1.97	**-2.46**
aCL1Contig17	No annotation available		0.02	-1.16	**-2.39**	**-2.03**	0.49	0.46	-0.38	-1.36
aC31504C04EF	Pollen Ole e 1 allergen and extension family protein	AT4G08685	-0.56	-0.81	**-2.28**	**-2.01**	-0.45	-0.95	-1.96	-1.90
aCL939Contig3	No annotation available		-0.29	-0.04	-0.82	-0.50	-0.78	-1.11	-1.75	**-2.27**
aC04006E11SK	Flavin reductase-related	AT2G34460	-0.28	**-2.10**	-1.63	-1.28	-0.55	**-2.21**	**-2.24**	-1.62
aCL533Contig3	CP12 precursor	AT3G62410	-0.29	-1.64	-1.79	-1.65	-0.77	-1.37	-1.78	**-2.21**
aCL5404Contig1	Hypothetical protein	AT5G38050	-0.15	**-2.21**	-1.35	-1.39	-0.12	**-2.32**	**-2.15**	-1.42
aCL3235Contig1	Expressed protein	AT3G62370	0.09	-1.88	-1.40	-0.98	-0.24	**-2.18**	**-2.12**	-1.34
aCL381Contig1	Galactinol synthase	AT1G56600	-0.96	-0.09	-	-0.55	-1.14	-0.52	-0.98	**-2.11**
aCL3Contig33	No annotation available		0.22	-0.84	-1.22	-0.74	-0.25	-0.94	-1.41	**-2.10**
aC16015G06SK	Expressed protein	AT3G01370	-0.12	**-2.03**	-1.57	-1.01	-0.36	**-2.05**	**-2.05**	-1.32
aCL310Contig2	Expressed protein	AT4G26850	-0.07	-1.60	**-2.04**	**-2.03**	-0.14	-1.26	-1.86	-1.93
aC04023D07SK	No annotation available		-0.22	-1.92	-1.55	-1.07	-0.29	-1.97	**-2.04**	-1.43

Table 4. Gene ontology (GO) biological process categories over-represented in the clusters based on the gene-to-gene correlation matrix (Fig. 2). Only three clusters (b, f and g) showed significant biological processes with Bonferroni multi-test adjustment (p<0.05).

Cluster	GO Term	Description	GO level	FDR
b	GO:0006950	response to stress	3	0.021
	GO:0009628	response to abiotic stimulus	3	0.034
f	GO:0019438	aromatic compound biosynthetic process	5	0.0041
	GO:0044249	cellular biosynthetic process	4	0.0055
	GO:0006519	cellular amino acid and derivative metabolic process	4	0.0087
	GO:0009058	biosynthetic process	3	0.0094
	GO:0044283	small molecule biosynthetic process	4	0.017
	GO:0006725	cellular aromatic compound metabolic process	4	0.026
	GO:0008610	lipid biosynthetic process	4	0.026
	GO:0044281	small molecule metabolic process	3	0.027
	GO:0019748	secondary metabolic process	3	0.030
g	GO:0044283	small molecule biosynthetic process	4	3.10E-11
	GO:0006519	cellular amino acid and derivative metabolic process	4	3.70E-09
	GO:0044281	small molecule metabolic process	3	2.10E-07
	GO:0006520	cellular amino acid metabolic process	5	7.40E-07
	GO:0044106	cellular amine metabolic process	5	1.70E-06
	GO:0009308	amine metabolic process	4	3.70E-06
	GO:0019752	carboxylic acid metabolic process	6	1.00E-05
	GO:0043436	oxoacid metabolic process	5	1.00E-05
	GO:0006082	organic acid metabolic process	4	1.10E-05
	GO:0008652	cellular amino acid biosynthetic process	6	1.10E-05
	GO:0006790	sulfur metabolic process	4	1.40E-05
	GO:0042180	cellular ketone metabolic process	4	1.50E-05
	GO:0042398	cellular amino acid derivative biosynthetic process	5	1.90E-05
	GO:0009309	amine biosynthetic process	5	2.70E-05
	GO:0034641	cellular nitrogen compound metabolic process	4	3.20E-05
	GO:0019438	aromatic compound biosynthetic process	5	3.60E-05
	GO:0046394	carboxylic acid biosynthetic process	6	3.80E-05
	GO:0016053	organic acid biosynthetic process	5	3.80E-05
	GO:0000096	sulfur amino acid metabolic process	6	6.50E-05
	GO:0006575	cellular amino acid derivative metabolic process	5	0.00015
	GO:0044271	cellular nitrogen compound biosynthetic process	5	0.00025
	GO:0006555	methionine metabolic process	7	0.00033
	GO:0009058	biosynthetic process	3	0.00034
	GO:0046483	heterocycle metabolic process	4	0.00042
	GO:0044249	cellular biosynthetic process	4	0.00044
	GO:0006725	cellular aromatic compound metabolic process	4	0.00089
	GO:0009066	aspartate family amino acid metabolic process	6	0.0028
	GO:0019748	secondary metabolic process	3	0.0073
	GO:0009699	phenylpropanoid biosynthetic process	7	0.015
	GO:0044237	cellular metabolic process	3	0.029
	GO:0009698	phenylpropanoid metabolic process	6	0.036
	GO:0008152	metabolic process	2	0.04
	GO:0006807	nitrogen compound metabolic process	3	0.043

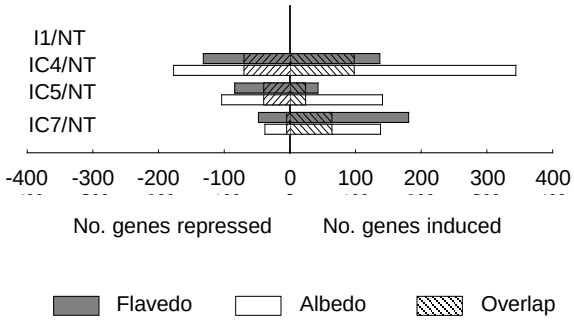

Fig. 1 (Ballester et al., 2011)

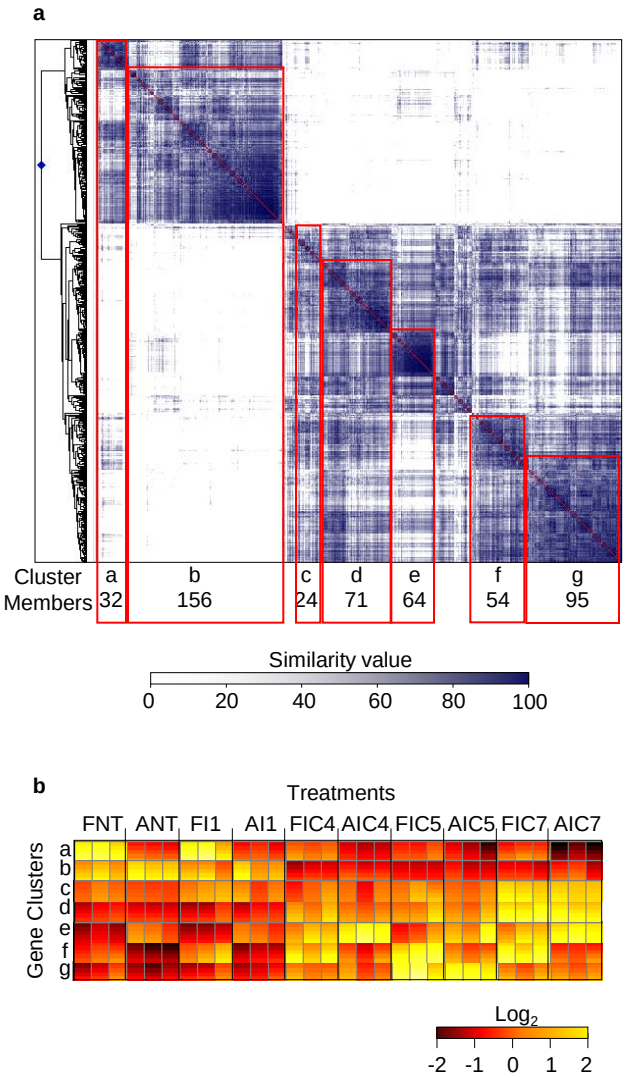

Fig. 2 (Ballester et al., 2011)

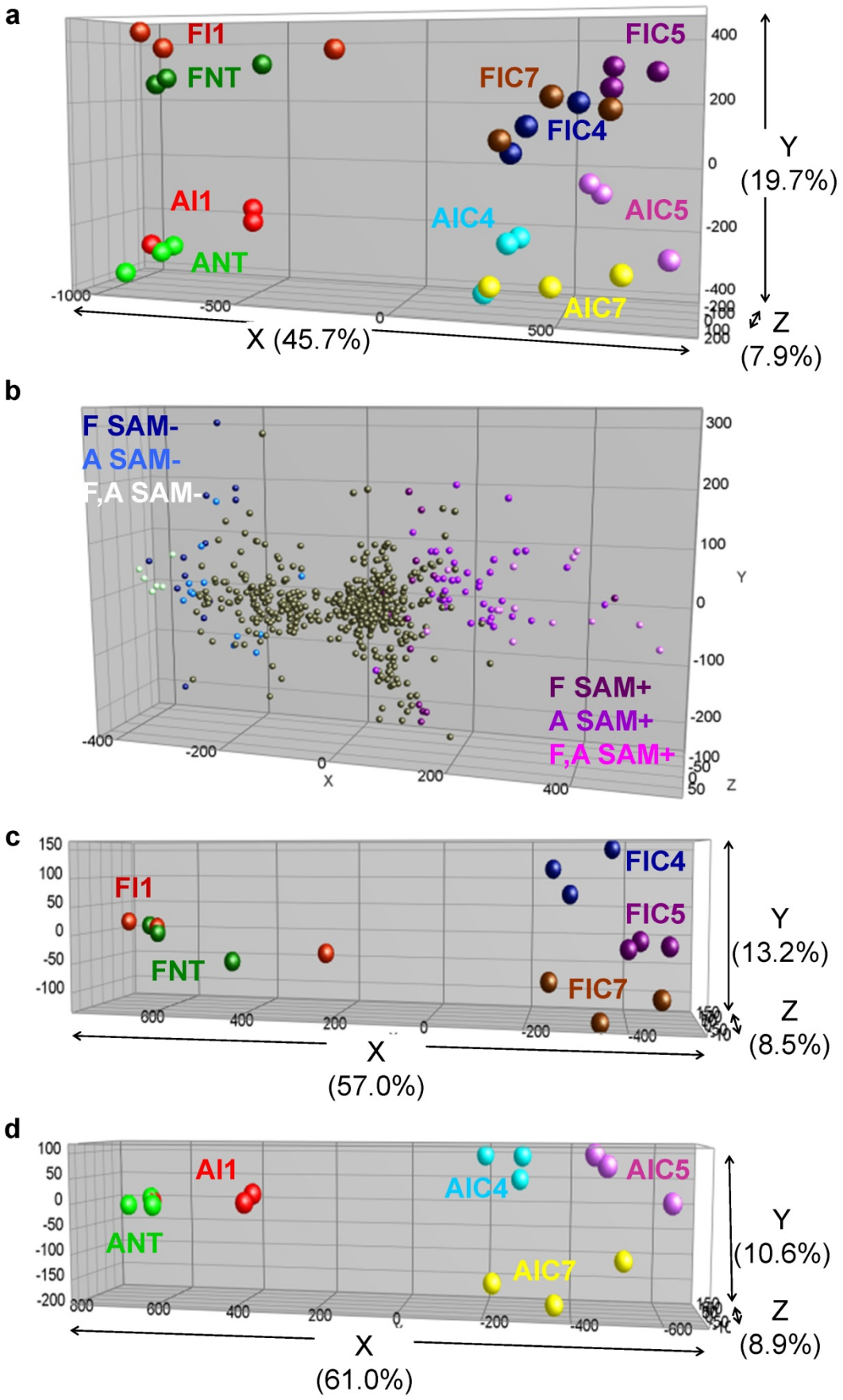

Fig. 3 (Ballester et al., 2011)

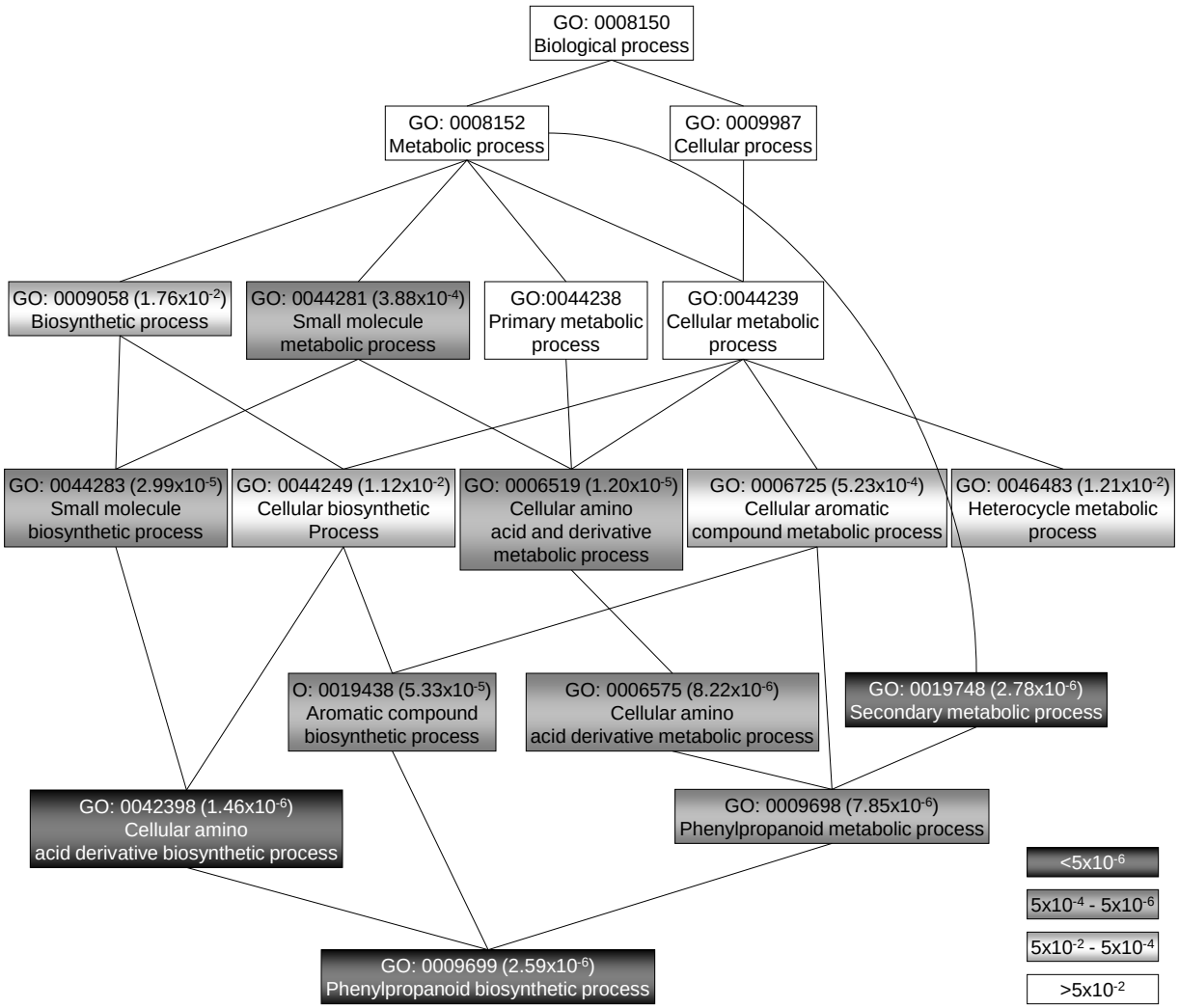

Fig. 4 (Ballester et al., 2011)

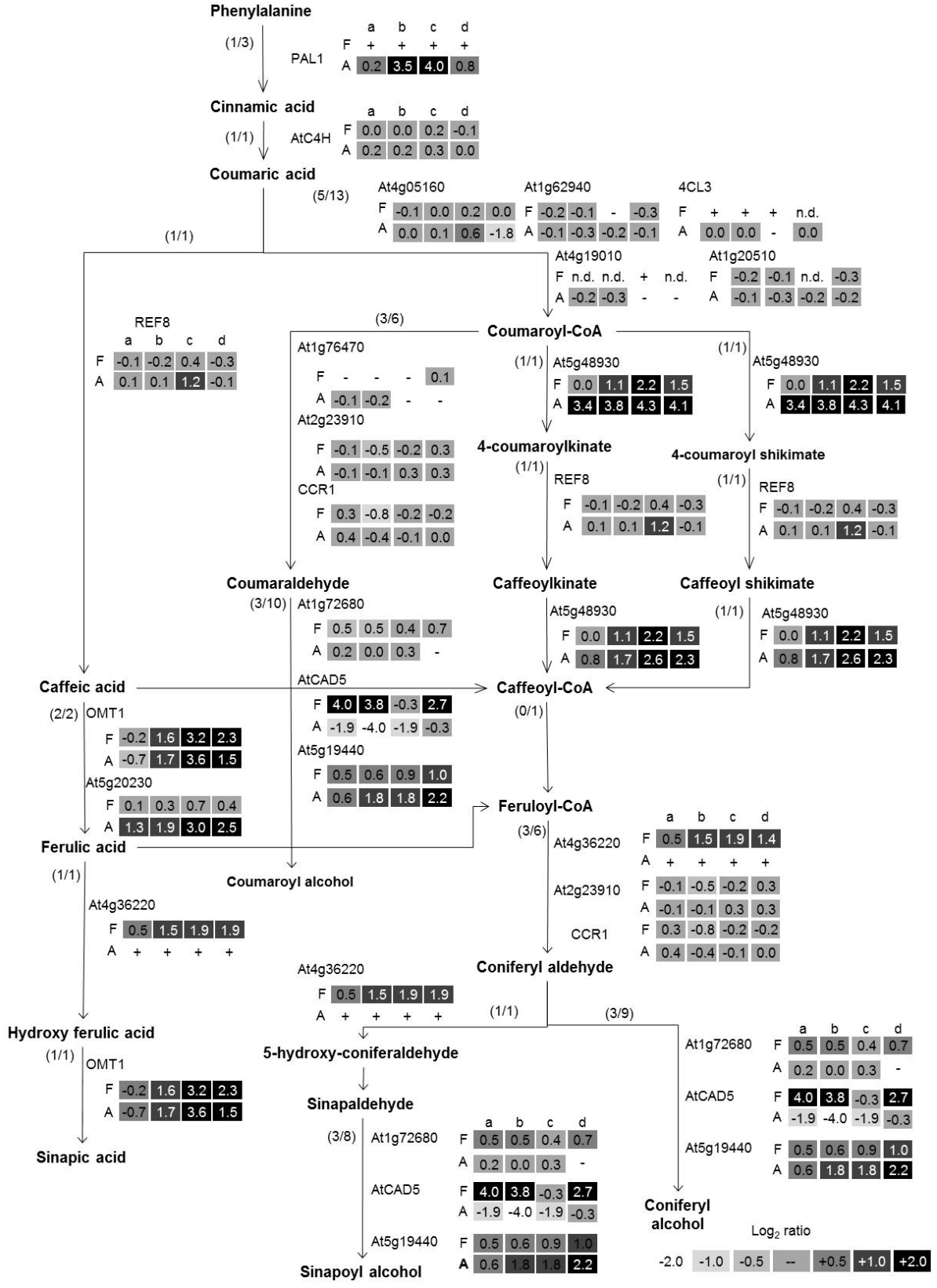

Fig. 5 (Ballester et al., 2011)

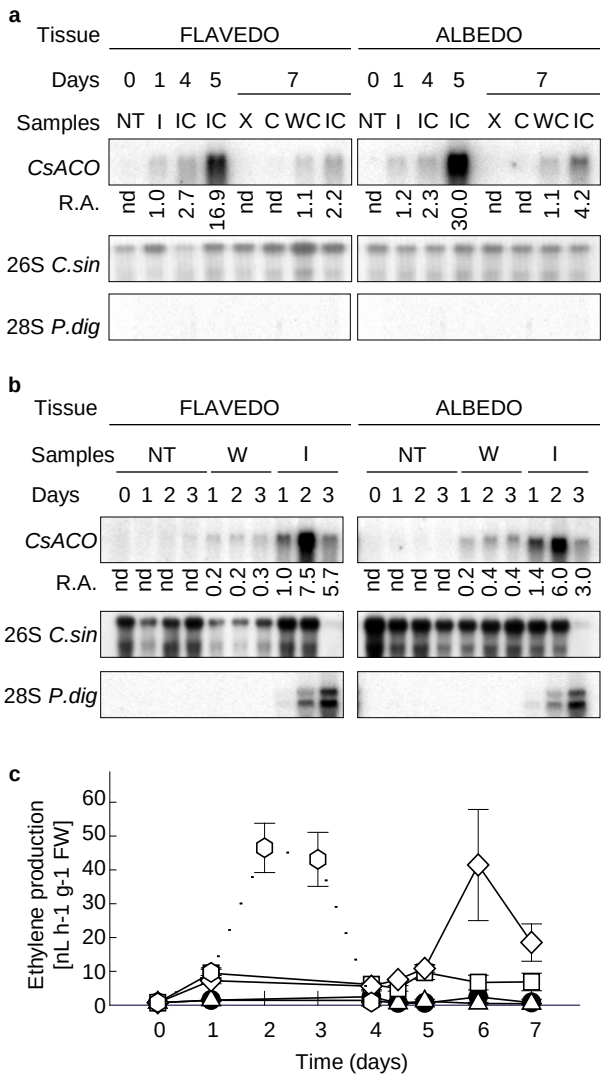

Fig. 6 (Ballester et al., 2011)

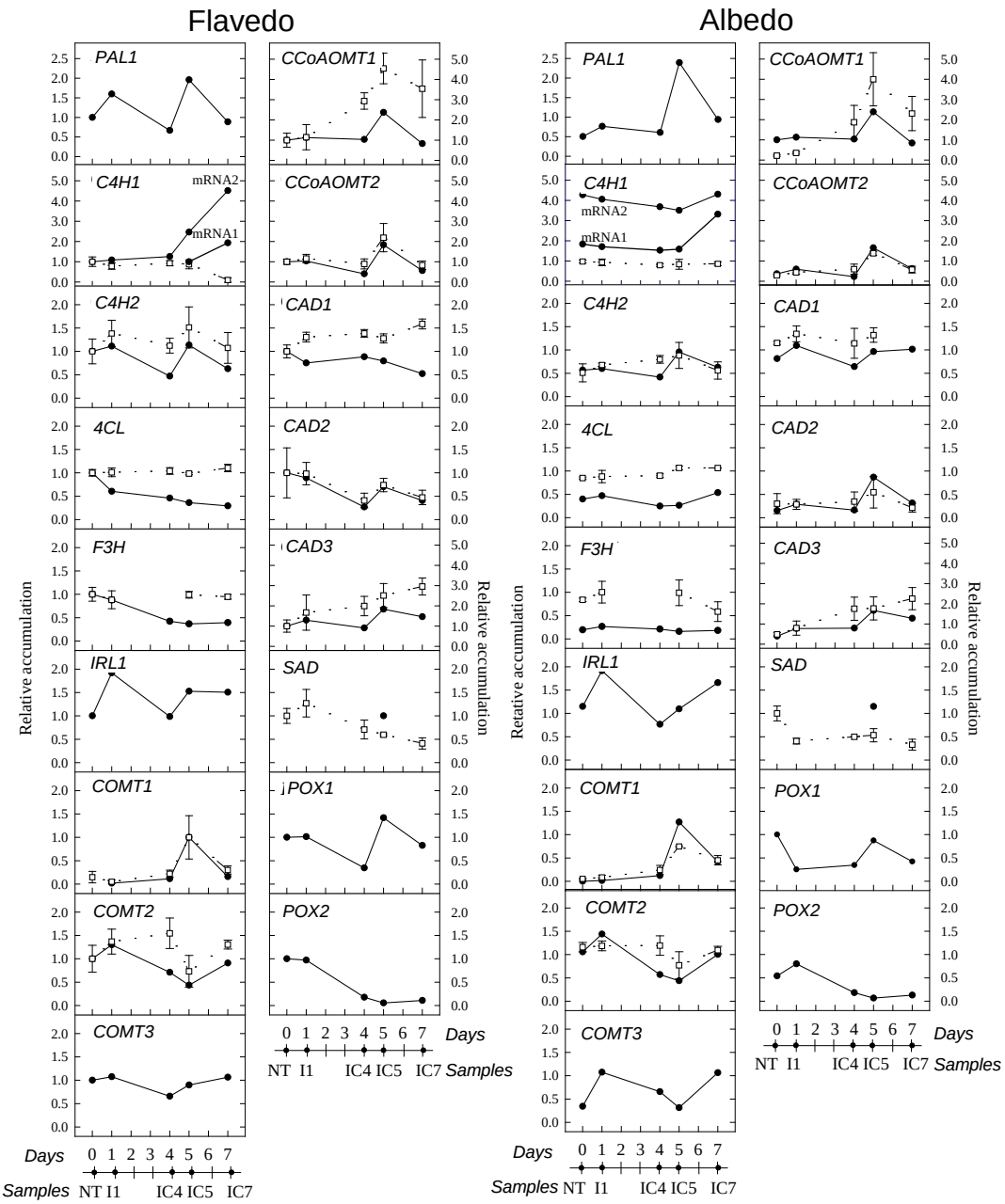

Fig. 7 (Ballester et al., 2011)